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13. ABSTRACT (Maximum 200 Words)

Flavopiridol is an investigational drug undergoing Phase II clinical trials for the treatment of various solid tumors. This drug has been shown to inhibit of the cyclin-dependent protein kinases, which are essential mediators of the cell division cycle. While this activity is likely to be responsible for its anticancer activity, it is nevertheless unclear how this drug achieves selective toxicity. To address this question, we created a clavopiridol-resistant cell line. The experiments described in this proposal are designed to identify the mechanism(s) responsible for rendering this cell line drug-resistant. Levels of cyclin-dependent protein kinase activity in this resistant cell line will be determined, and compared to those seen in the parental, drug-sensitive cell. In addition, levels of relevant drug-detoxifying enzymes, such as UDP-glucoronosyl transferase and glutathione S-transferase will be measured in these two cell lines, as will levels of drug transporters. Additional strategies will examine whether other gene products are over or under expressed in drug-resistant cells, as well. It is anticipated that the results of these studies will shed light on the mechanism of flavopiridol resistnance. This information could be of value in designing second generation drugs that may prove more effective in the treatment of cancer.

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New anticancer drugs, flavopiridol, drug resistance, cross-resistance, molecular determinants of cellular sensitivity to cytotoxic agents, cell cycle kinetics, cell culture, cloning

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Introduction

Flavopiridol (FP) is an investigational new drug currently in Phase II clinical trials for the treatment of solid tumors (Senderowicz *et al.*, 1998). The precise mechanism of action of this compound is unknown, however, it is known to inhibit several members of the cyclin-dependent protein kinase family, and to induce cell death (Senderowicz, 1999, Schrump, *et al.*, 1998, Bible and Kaufmann, 1996). The objective of this proposal is to gain insight into the molecular mechanism(s) whereby human tumor cells become resistant to FP. To gain greater insight into this question, a FP-resistant clone was generated from the human MCF-7 breast adenocarcinoma cell line. This clone was obtained by exposing growing cultures of MCF-7 cells to increasing concentrations of FP over a several month period of time. The resulting clone, named MCF-7/F, has an IC_{50} for FP that is 24-fold lower than that of the parental MCF-7 cell line cell line from which it was derived. Specifically, the experiments described in this proposal are designed to identify the molecular basis for FP resistance in MCF-7/FP cells. We have evaluated, or are in the process of evaluating cellular levels of known FP targets, i.e. cyclin-dependent protein kinases in MCF-7/FP and MCF-7 cells. In addition, we have begun to examine the relative resistance of these cells to a number of drugs. Finally, we have been examining the relative expression levels of a number of drug efflux pumps in these cell lines. It is anticipated that FP or FP-like molecules will ultimately assume a place in the modern cancer chemotherapeutic armamentarium. Thus, insight gained into the molecular basis of FP drug resistance in MCF-7/FP cells may ultimately prove beneficial in the design of second or third generation FP analogues. It is also conceivable that this information may aid in the development of chemotherapy strategies to minimize the emergence of clinical resistance to these agents.

Body

Task 1. Ascertain whether human normal breast epithelial MCF-10A cells are, relative to human breast adenocarcinoma MCF-7 cells, less sensitive to FP.

> As was discussed in the previous report, we find no evidence that MCF-10A cells are more resistant to FP than are MCF-7 cells. This task is considered completed.

Task 2. Ascertain whether stable resistance to FP on the part of MCF-7/FP cells persists beyond 90 cell divisions.

> As was discussed in the previous report, we find that the MCF-7/FP cells are stably resistant to FP. This task is considered completed.

Task 3. Ascertain whether the sensitive MCF-7 cell line and the insensitive MCF-7/FP subline differ in selected cell cycle parameters.

> As was discussed in the previous report, we find that the MCF-7 and MCF-7/FP cells differ slightly in their cell cycle parameters. The MCF-7-FP cells were shown to display a distribution across the various phases of the cell cycle that differed from that of the MCF-7 cells. In particular, the former cells were far

more likely to be in the S phase of the cell cycle, and less likely to be in the G0-G1 phase of the cell cycle, compared to the latter cells. Interestingly, when a similar analysis was performed on MCF-7 and MCF-7/FP cells from stationary phase cultures, both cell types displayed nearly identical distributions. It is noteworthy that the cell cycle parameters of the MCF7 cells were similar whether they were measured in mid-log-phase growth cultures or stationary phase cultures. The basis for this phenomenon is not clear, however, we have not yet been able to gain additional insight into the underlying mechanisms.

This task is considered completed.

Task 4. Ascertain whether MCF7/FP cells are cross-resistant to flavopiridol analogues, other flavone anticancer agents, UCN-01, and/or anticancer agents presently used to treat metastatic breast cancer.

We have examined the relative cross-resistance of MCF-7/FP cells to a variety of agents. These results are presented in Figure 5 of the manuscript Lakshmipathy et al in the appendix.

This task is considered completed.

Task 5. Ascertain whether elevated levels of the kinase(s) inhibited by FP account for MCF-7/FP insensitivity to this agent.

As was outlined in the previous annual report, we have completed this analysis, the results of which are presented in Figure 2 of the manuscript Lakshmipathy et al in the appendix.

This task is considered completed.

Task 6 Ascertain whether an elevated level of a drug transport pump, a drug metabolizing enzyme and/or a nontarget flavopiridol-binding protein accounts for MCF-7/FP insensitivity to flavopiridol.

As was outlined in the previous annual report, we have completed this analysis, the results of which are presented in Figure 4 of the manuscript Lakshmipathy et al in the appendix.

We do not believe that the drug resistance profile of MCF-7/FP cells can be explained by overexpression of any currently identified drug efflux pump. Thus, we hypothesize that the FP drug resistance associated with MCF-7/FP cells is due to over expression of a novel drug transporter molecule. We have initiated a series of experiments to test whether gene transfer can be used to confer FP resistance on a sensitive donor cell line. If these experiments prove successful, we will attempt to functionally clone the gene responsible for conferring resistance. Our

working hypothesis is that this gene will encode a novel drug efflux pump. It is anticipated that these experiments will require many months, or even years to complete. We have not made significant progress towards this objective since submission of the previous report.

Task 7. As was outlined in the original proposal, this task will only be pursued if completion of Tasks 5 and 6 do not provide a full phenotypic explanation for the acquired resistance of the MCF-7/FP cells. Thus we have no plans to pursuing this task at the current time.

Key Research Accomplishments. Results obtained during the period of support of this proposal permit us to reach the following conclusions.

1. The drug resistance seen in the MCF-7/FP cells is stable.
2. The cell doubling time of the MCF-7/FP cells does not differ significantly from that of the parental MCF-7 cells. However, the distribution of the two cell lines within the distinct phases of the cell cycle does appear to differ, particularly when cells are rapidly dividing.
3. MCF-7/FP cells display a unique pattern of drug cross-resistance. They are modestly resistant to topotecan and mitoxantrone, an are not resistant to cisplatin, paclitaxel or vinblastin.
4. MCF-7/FP cells do not display altered levels of the cdks 1, 2, 4, 7, 8, or 9.
5. MCF-7/FP cells do not display altered levels of cyclins A, B1, D3 or E. They do have slightly reduced levels of cyclin D1.
6. MCF-7/FP cells have an approximate three-fold increase in the level of Abcg2 mRNA, and protein, compared to MCF-7 cells.
7. MCF-7/FP cells do not have elevated levels of the MDR-1 protein.
8. The FP resistance of MCF-7/FP cells does not appear to be due to the modestly elevated levels of Abcg2 protein, however, these cells do display elevated ATP-dependent efflux of lysotracker green, suggesting that they do overexpress an unknown drug efflux pump.
9. Taken together we propose that the drug resistance phenotype of MCF-7/FP cells results from overexpression of a novel drug efflux pump.

Reportable Outcomes. The manuscript Lakshmipathy et al., (APPENDIX) was submitted for publication. This manuscript is similar to that which was provided along with last year's annual report, although it was somewhat revised prior to submission. Reviews were generally favorable, yet the editor wishes us to perform an addition series of experiments. We are currently initiating this work, and hope to submit a revised manuscript within the next 2-3 months. An abstract of this work was presented at the Era of Hope Department of Defense Breast Cancer Research Program (P 55-4). A copy of the abstract is included as part of the APPENDIX.

Conclusions. There appear to be numerous mechanisms through which cells can become resistant to the cytotoxic effects of FP. It has been well established that overexpression of the ABCG gene may lead to resistance (Robey *et al.*, 2001). However, the drug

resistance profile of other FP-resistant cell lines suggests that their resistance to FP may not be due to a similar mechanism, since they failed to detect any evidence for reduced FP accumulation in this cell line (Smith *et al.*, 2001). In addition, Bible *et al.* (2000) described an ovarian cell line that appears to be resistant to FP through yet another mechanism. In contrast to the cell lines described by Robey *et al.* and Smith *et al.*, (and that which we have described) the ovarian cancer cell line acquired FP resistance spontaneously. In addition to being FP resistant, these ovarian cells were also resistant to cisplatin, a phenotype not associated with the other FP-resistant cell lines. Furthermore, these cells were not resistant to mitoxanthrone. Thus, while direct evidence is lacking, these findings suggest that the mechanism through which these ovarian cancer cells became resistant to FP, although apparently due to reduced intracellular accumulation of drug, is nevertheless unlikely to have resulted from overexpression of the ABCG2 gene.

The phenotype of the MCF-7/FP cells suggests that the mechanism through which they have become FP resistant is distinct from that associated with the FP-resistant cell lines discussed above. While overexpression of the ABCG2 transporter is seen in the MCF-7/FP cells, the level of overexpression is far lower than that described by Robey *et al.* This finding is particularly intriguing since the Robey group used an essentially identical protocol to generate the drug-resistant MCF-7 derivative cell line (which they called MCF-7 FLV1000). Robey *et al.* used RT-PCR to determine that the MCF-7 FLV1000 cells had a nearly 50-fold increase in ABCG2 mRNA levels, compared to the parental MCF-7 cell line. (Inspection of the northern blot data presented in their paper suggests this may actually be a minimal estimate of the degree of overexpression of this gene.) In contrast, the northern and western blot analysis of RNA from the MCF-7/FP and MCF-7 cells described herein indicates that levels of ABCG2 gene expression in the former are elevated no more that 3-fold over those seen in the latter. Perhaps most importantly, we find that the ABCG2 inhibitor fumitremorgin C fails to sensitize the MCF-7/FP cells to FP.

These preceding discussion highlights that resistance to FP can occur *via* multiple mechanisms. Perhaps more provocatively, these results suggest that multiple mechanisms of FP resistance may function within a single cell. For example, the data included in this report are consistent with the conclusion that while overexpression of the ABCG2 drug transporter contributes to the FP resistance seen in MCF-7/FP cells, there are likely to be additional drug resistance mechanisms at work in these cells. This finding is of great significance. It implies that not only may that clinical resistance to FP arise via multiple mechanisms amongst different patients, but that within a single patient there may be multiply resistant cancer cells. If FP or FP analogs are to eventually become important anticancer agents, it is imperative that the mechanisms through which these distinct resistance pathways function be fully understood.

The availability of the MCF-7/FP cell line will permit us to address this issue in greater detail. We are particularly enthusiastic about the prospect of using the MCF-7/FP cells as a donor in gene transfer experiments designed to confer resistance to flavopiridol on an inherently sensitive recipient cell line. These experiments hold the promise of helping to identify novel mechanisms of drug resistance. This information, in turn may ultimately play an important role in efforts to understand to molecular basis of drug resistance in cancer cells, thereby paving the way to novel therapeutics with which to fight this disease.

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GENERATION AND CHARACTERIZATION OF A MCF7 CELL LINE RESISTANT TO FLAVOPIRIDOL WITH A UNIQUE PATTERN OF DRUG CROSS-RESISTANCE

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Key Words: Flavopiridol, MCF7, Drug Resistance, ABCG2 transporter, cell cycle

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Classification: Chemotherapy and metabolic inhibitors

SUMMARY

Molecular characterization was performed on a MCF7-derived clone selected on the basis of its resistance to the experimental anticancer agent flavopiridol. The resistant clone (MCF7/FP), which was obtained by serial passage of MCF7 breast cancer cells through increasing concentrations of flavopiridol, was more than 25-fold less sensitive to the cytotoxic effects of this drug than was the parental cell line. Western blot analysis revealed that cdks 1, 2, 4, 7, 8, and 9 were expressed at equivalent levels in the MCF7/FP and parental MCF7 cells. Likewise, expression levels of cyclins A, B, D3 and E were indistinguishable in the two cell lines, however the MCF7/FP cells had slightly diminished levels of cyclin D1 protein, compared to the parental MCF7 cells. The level of the ABCG2 membrane transport protein was slightly elevated in MCF7/FP cells, compared to parental MCF7 cells. However, there are several reasons why elevated ABCG2 expression is unlikely to be responsible for the flavopiridol resistance seen in MCF7/FP cells. First, modest increases in ABCG2 expression levels do not confer resistance to high levels of flavopiridol-resistance to MCF7 cells. Second, MCF7/FP cells were not cross-resistant to mitoxantrone or topotecan, as is seen in MCF7 cells that overexpress the ABCG2 protein. Finally, treatment of MCF7/FP cells with fumitremorgin C, a potent inhibitor of ABCG2, did not sensitize the cells to flavopiridol. The resistance to flavopiridol observed in MCF7/FP cells instead appears to result from elevated levels of an as yet uncharacterized ATP-dependent drug efflux pump.

Key words: chemotherapy, drug-resistance, efflux pump, flavopiridol, MCF7 cells

INTRODUCTION

Flavopiridol (FP) is a semisynthetic flavone derivative of the alkaloid rohitukine present in the bark of the Indian tree *Dysoxylum binefacterium*. It is the first potent cyclin dependant kinase inhibitor drug to undergo clinical trials in the United States (1, 2, 3). FP is known to specifically inhibit cdks 1,2,4 and 7 with equal potency by competing for the ATP binding sites of these kinases (4-7). FP is also known to down-regulate the expression of cyclins D1 and D3, which are required for the activation of cdk4 and cdk6, respectively (8, 9). Consistent with this observation, flavopiridol induces cell cycle arrest in breast and lung cancer cells *in vitro* (4, 6, 10, 11). Apart from this cytostatic effect, FP exposure induces apoptosis in both resting and proliferating cells (12). The induction of apoptosis was found to be independent of the p53 and p16 status of the cells (13, 14). Upon completion of phase I clinical trails (15), phase II trials have been initiated along with combination studies evaluating the efficacy of co-administration of paclitaxel and cisplatin, since a synergy between these drugs was demonstrated (11).

A major limitation to use of anticancer drugs in clinical setting is the acquisition of drug resistance by the cancer cells. Multiple mechanisms are associated with drug-resistant cancer cells including: overexpression of drug transporters resulting in decreased intracellular drug concentration, altered metabolism of the drug, increased DNA repair, and modification of the drug target itself (16). Even if FP fulfills its original promise as an antineoplastic agent, its clinical efficacy is likely to be limited due to the emergence of drug-resistance.

Several cell lines have been reported with acquired resistance to FP on long exposure. An ovarian cancer cell line resistant to cisplatin and FP has been reported. The mechanism of resistance has not been clearly established (17). A human colorectal carcinoma cell line with acquired resistance to FP was recently reported (18). Again, the mechanism through which this clone acquired FP-resistance remains obscure. Recently, it has been established that overexpression of the ATP-binding cassette half-transporter ABCG2 confers on MCF7 cells a FP-resistant phenotype (19-21). The ABCG2 transporter is identical to previously reported BCRP, ABCP1, and MXR (22, 23), Overexpression of this protein also confers resistance to mitoxantrone, antracyclins, topotecan and SN-38 (7-ethyl-10-hydroxy-camptothecin).

We describe a MCF7-derived cell line that was isolated following prolonged exposure to escalating concentrations of FP. This cell line, named MCF7/FP, displays modestly elevated levels of the ABCG2 drug transporter protein. However, previous results indicate that this level of expression by itself is not sufficient to provide the levels of drug resistance observed in these cells. Furthermore, the spectrum of cross-resistance observed in MCF7/FP cells differs from that reported previously in cells that overexpress ABCG2. Finally, treatment of MCF7/FP cells with the ABCG2 transporter inhibitor fumitremorgin C failed to sensitize them to FP. Nevertheless drug efflux studies suggest that FP resistance in MCF7/FP cells is due to elevated levels of a previously uncharacterized ATP-dependent drug efflux pump.

MATERIALS AND METHODS

Chemicals: The drug synthesis and chemistry branch of the National Cancer Institute provided FP. All cdk and cyclin antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. BCRP antibody was purchased from Kamiya Biomedical Company, Seattle, WA, and anti-P gycoprotein antibody from Calbiochem, La Jolla, CA. Fluorescent dyes Rhodamine 123 and Lysotracker green were purchased from Molecular Probes Inc., Eugene, OR. Cisplatin and Calcein AM were purchased from Sigma Chemical Company, St. Louis, MO; Vinblastine and Paclitaxel from ICN Biochemicals, Costa Mesa, CA; and Topotecan from LKT Laboratories Inc., Saint Paul, MN. Dulbeco's modified Eagle's media (DMEM) was purchased from Gibco BRL. Fumitremorgin C was provided by Dr Lee Greenberger, Wyeth-Ayerst Research, Pearl River, NY. All other chemicals were from the Sigma Chemical Company unless otherwise specified. MCF7 cells were obtained from A. M. Senderowicz, National Cancer Institute, Bethesda, MD.

Isolation of FP-resistant MCF7 cell line: A derivative of the human breast cancer cell line MCF/7 was expanded and maintained in DMEM supplemented with 10% equine serum and 50 µg/ml gentamycin. These cells were treated with 0.2mM FP for 48 hours, rinsed with fresh growth media lacking the drug and allowed to grow for an additional 48–72 h. Cells were harvested, sub-cultured and the drug treatment described above repeated. The concentration of the drug was increased once every month. The concentration of FP was thus gradually increased up to a final concentration of 10 mM and the cell thus isolated were named MCF7/FP. MCF7/FP cells remained fully resistant to FP even following three months of culture in the absence of drug.

Whole cell extracts: Parental MCF7 cells and MCF7/FP were grown to confluence. Cells were scraped with a rubber policeman, washed thrice with phosphate buffered saline and suspended in 10 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$, 10 mM KCl, 1 mm DTT and protease inhibitors. The samples were incubated on ice for 20 min and subjected to 20 strokes in a dounce homogenizer to release the nuclei. Sodium chloride was added to the homogenized cells to a final concentration of 350 mM and incubated on ice for 1 hour to allow lysis of the nuclei by hypoosmotic shock. The cell extract was then centrifuged at 70,000 RPM in a Beckman TL-100 rotor at 2 °C for 30 min to remove the membrane fraction. The clear supernatant representing the whole cell lysate was collected and protein concentration determined by Coomassie brilliant blue G-250 dye-binding (24).

Preparation of Plasma Membrane: Monolayers of confluent cells were washed with cold PBS and scraped using a rubber policeman. Cells were suspended in a 10mM HEPES buffer containing 2mM EDTA, 100mM NaCl, 1mM DTT and protease inhibitors. The cells were homogenized as described above and the cell debris, nuclei and unbroken cells removed by centrifugation at 3000 rpm for 10 min in a refrigerated tabletop centrifuge. The supernatant was collected and plasma membranes isolated by centrifugation at 70,000 RPM for 30 min in a Beckman TL-100 rotor. The plasma membrane pellet was resuspended in 2% (w/v) SDS solution containing 1mM DTT and protease inhibitors. Aliquots of the membrane fraction were precipitated by trichloroacetic acid and the protein concentration determined by the method of Lowry (25).

Western blot Analysis: Thirty µg of whole cell extracts or 50-100 µg of plasma membrane preparations from MCF7 and MCF7/FP cells were resolved on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked in TRIS buffered saline containing 0.1% bovine serum albumin for a period of 1 h at room temperature. Blots were incubated with primary antibodies diluted to concentrations recommended by the vendor at 4 °C overnight. Following incubation with primary antibody, blots were washed thrice with TRIS buffered saline containing 0.01% (w/v) bovine serum albumin and treated with alkaline phosphate conjugated secondary antibody diluted 1:5000 at room temperature for 1 h with constant agitation. Blots were then washed thrice with TRIS buffered saline containing 0.01% bovine serum albumin and developed using the alkaline phosphate substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Northern Blot Analysis: Cytoplasmic RNA was isolated from cells as described previously (26). 10 µg of RNA from MCF7 and MCF7/FP cells was resolved by electrophoresis on an agarose gel and transferred to a nylon membrane by capillary action. Membranes were prehybridized at 60 °C in Church Buffer (27) for 1 h and hybridized overnight at 60 °C with a portion of the ABCG2 cDNA labeled with [α-^{32}P] dATP using the random prime kit (Gibco BRL, Grand Island, NY).

Cytotoxicity Assays: The effects of various cytotoxic drugs on the survival of MCF7 and MCF7/FP cells were analyzed by either clonogenic assay or by the sulforhodamine B method. ***Clonogenic Method***-Colony forming assays was performed by plating 6000 cells per Petri dish in multiple 10cm dishes containing DMEM supplemented with 10% equine serum and 50 µg/ml gentamycin. The cells were allowed to grow for 24 hours.

Drugs (dissolved in DMSO or methanol) or equivalent volumes of vehicle were added to cells, and incubated for 24 h. The cells were subsequently washed thrice with serum-free media, fresh media added to the cells, and the cultures allowed to grow for an additional 12-14 days. The resulting colonies were stained with crystal violet and counted. Three independent experiments were carried out and the average number of colonies formed at each drug concentration determined and used to calculate the percent survival. ***Sulforhodamine B (SRB) method:*** Cytotoxicty was also measured using the sulforhodamine B method as described previously (28). Briefly, 100,000 cells were plated in 24-well plates in triplicate, and allowed to attach for 24 h. Adherent cells were exposed to varying concentrations of drug for 48 h. Following this period, cells were washed thrice and allowed to recover for 24 hours in serum-containing media. Cells were fixed in 10% TCA and stained with 0.4% sulforhodamine B dissolved in 1% acetic acid. The absorption at 540 nm was measured and used to calculate relative percent survival.

Inhibition of ABCG2 by fumitremorgin C: Sensitivity of MCF7/FP cells to FP in the presence and absence of fumitremorgin C was examined by SRB and clonogenic methods as described above. Cells were treated with FP either in the absence or presence of 5 mM fumitremorgin C as described above.

Efflux Studies: Cells were grown on cover slips in a 10 mm dish to 70% confluence. The fluorescent dyes, Lyostracker green, Calcein AM and Rhodamine 123 were added to a final concentration of 1 mM each and incubated at 37 °C in a CO_2 incubator for 30 min. Cover slips were washed three times with PBS and fresh media added to the coverslips in 10 cm dishes. Cells were immediately observed under a confocal

microscope. In some experiments cells grown on cover slips were incubated for 1 h at 37 °C in the presence of 15 mM sodium azide and 50 mm deoxyglucose prior to addition of fluorescent dye.

Confocal Microscopy: Cells grown on coverslips and incubated with fluorescent dyes were scanned using a Bio-Rad MRC-1024 confocal system. A 60X-water immersion objective was used, and the samples were excited at 488 nm and emission monitored at 522 nm. Digitized fluorescence images were analyzed with the aid of Adobe Photoshop.

RESULTS

MCF7/FP cells are resistant to FP: MCF7 cells were grown in serially increasing concentrations of FP and a cell line, hereafter referred to as MCF7/FP, was generated. A clonogenic assay was performed to measure the sensitivity of parental MCF7 and MCF7/FP to the cytotoxic effects of FP. As Figure 1 indicates, MCF7/FP cells were significantly more resistant to the cytotoxic effects of FP than were the parental MCF7 cells. The LC50 of FP for MCF7/FP cells was 0.3 mM, which is 60-fold higher than that for the parental MCF7 cells (LC50=0.005 mM). The LC90 values were 0.18 mM for MCF7 cells and > 8 mM for MCF7/FP cells.

Comparison of cell cycle regulation protein levels: The mechanism of action of FP is thought to involve inhibition of cell cycle regulation proteins such as cdks and cyclins (See Introduction). Since alteration in the levels of these cell cycle regulation proteins could overcome the inhibitory effect of FP, the levels of cdks and cyclins were examined in the MCF7 and MCF7/FP cells. Whole cell extracts were prepared from MCF7 and MCF7/FP cells and western blot analysis performed using a variety of antibodies. Figure 2A shows representative western blots obtained with antibodies specific for cdks 1, 2, 4, 7, 8 and 9. All analyses were repeated with three independently prepared extracts and intensity of bands corresponding to the respective cdks quantified. The densitometry data was used to determine the relative levels at which each of the cdk proteins were expressed in the two cell lines. The mean ratio values obtained ($\pm$ the standard error of the mean) are represented in the bar graph depicted in Figure 2B. Statistical analysis revealed that in no case were the differences between cdk levels in the two cell lines statistically significant ($p>0.05$, student t test).

The relative levels of expression of 5 different cyclin proteins in MCF7 and MCF7/FP cells were also measured. Figure 3A depicts the results from a representative experiment. The band intensities were again quantified from western blots performed on three independently prepared sets of whole cell extracts and the ratio of protein expression levels in the MCF7/FP cells versus those present in MCF7 cells were plotted (Figure 3B). This analysis reveals no significant difference in the respective levels of cyclins A, B, D3 and E in the two cell lines. In contrast, the amount of cyclin D1 protein present in extracts from MCF7/FP cells was nearly 2-fold lower than that present in extracts from MCF7 cells. This difference in cyclin D1 protein levels was statistically significant ($p=0.045$, student t test).

Drug Transporters: Northern blots analysis was performed on total RNA isolated from MCF7 and MCF7/FP cells. As depicted in Figure 4A (Left panel), ABCG2 mRNA expression levels were higher in MCF7/FP cells compared to MCF cells. Since the intensity of GAPDH band is comparable in both samples, this difference is not due to unequal loading. Northern blot analysis of three independent RNA preparations was carried out and the relative intensities of the bands corresponding to the ABCG2 and GAPDH mRNAs quantified. The ratio of the expression levels of these two mRNA species present in MCF7/FP cells versus MCF7 cells was determined. The ratio values presented in Figure 4A (right panel) indicate that the level of ABCG2 mRNA in the MCF7/FP cell line is elevated 3 to 4-fold above that present in MCF7 cells. In contrast, there is no appreciable difference between the levels of GAPDH mRNA expression in the two cell lines.

To determine if the increase in ABCG2 mRNA corresponded to elevated ABCG2 protein levels in MCF7/FP cells, western blot analysis was performed. Initial efforts to detect ABCG2 protein levels in whole cell extracts prepared from these cells failed to detect any signal (data not shown). Since the ABCG2 protein is localized within the plasma membrane, western blots analysis was then performed on plasma membrane fractions prepared from these cells. Duplicate sets of extracts were resolved by SDS-polyacrylamide gel electrophoresis. One portion of the gel was stained with Coomassie blue, and the remainder was transferred to a nitrocellulose membrane and probed with either anti-ABCG2 antibody, or as a control with anti-P-glycoprotein antibody. Examination of the Coomassie blue-stained SDS gel (Figure 4B) reveals that equivalent amounts of protein from the MCF7 and MCF7/FP cell lines had been loaded on the gel. Western blot analysis revealed that while both cells express similar levels of P-glycoprotein, ABCG2 protein is present at an elevated level in the MCF7/FP cells. Quantification of the band intensities obtained with three independent plasma membrane fractions was carried out as described above and the ratio of relative expression levels between the two cells plotted (Figure 4B, Right Panel). This analysis reveals an approximate 4-fold increase in the levels of ABCG2 protein in the extracts from the MCF7/FP cells, with no detectable difference in the level of p glycoprotein expression. The former result is similar to the approximately 3-fold elevation in the level of ABCG2 mRNA in these cells. We also performed immunocytochemistry to visualize the increased expression of ABCG2 protein in the MCF7/FP cells. Figure 4C depicts a representative fluorescence image of MCF7 (Left panel) and MCF7/FP (Right panel), treated with anti-ABCG2 antibody and a rhodamine-conjugated secondary mouse

antibody. As expected, increased fluorescence intensity was observed in the MCF7/FP cells, compared to the parental MCF7 cells.

Cross resistance of MCF7/FP to other cytotoxic drugs: The resistance of MCF7 and MCF7/FP to other drugs was examined. Figure 5 shows the cytotoxicity curves obtained for the MCF7 and MCF7/FP cells with five different drugs. Paclitaxel and vinblastine are drugs known to be effluxed by the p glycoprotein transporter while topotecan and mitoxantrone are effluxed by ABCG2 but also exhibit cross-resistance pattern with P-glycoprotein (21). Recently, an ovarian cancer cell line with cross-resistance to flavopiridol and cisplatin was identified (17). Therefore MCF7 and MCF7/FP were tested for cytotoxic effects of cisplatin. As Figure 5 demonstrates, both cell lines are equally sensitive to cisplatin, paclitaxel and vinblastine. The MCF7/FP cell line displayed a very slightly elevated resistance to topotecan and a 2.5-fold elevated resistance to mitoxantrone (LC50=2.6 μM, versus 1.0 μM for MCF7/FP and MCF7, respectively).

The cross resistance pattern obtained with the MCF7/FP cells is rather intriguing and is distinct from that observed for other ABCG2 overexpressing cell lines. Table 1 shows the IC50 values reported previously and the values obtained by the current study. MCF7 cells resistant to flavopiridol reports cross resistance of 100 to 2000 fold to mitoxantrone and 8 to 1500 fold to topotecan. Our study shows that the flavopiridol resistant cell line MCF7/FP is merely 2.7 fold more resistant to mitoxantrone compared to its parental MCF7 cells with no significant difference in the toxicity to topotecan.

Next, the sensitivity of MCF7/FP to flavopiridol in the presence and absence of fumitremorgin C, a potent inhibitor of ABCG2, was assessed. As Figure 6 shows, the resistance of MCF7 /FP cells to flavopiridol is identical in the absence or presence of

fumitremorgin C. Interestingly, fumitremorgin C did partially sensitize the cells to mitoxantrone, suggesting that ABCG2 overexpression does contribute in part to the resistance of MCF7/FP cells to this agent (data not shown).

Drug Efflux: Since ABCG2 overexpression might be expected to result in reduced intracellular drug accumulation, confocal fluorescence microscopy was used to examine MCF7 and MCF7/FP cells that had been exposed to different dyes. As Figure 7 indicates, there was no apparent difference in the intracellular concentrations to which calcein AM or rhodamine 123 accumulated in the two cell lines. In contrast, lysotracker green accumulated to significantly higher levels in MCF7 cells, compared to the MCF7/FP cells. Consistent with earlier reports (29), the efflux of lysotracker green was unaffected by the presence of fumitremorgin C indicating a lack of ABCG2 involvement in the efflux process of this drug (data not shown).

To test the hypothesis that the reduced cellular concentration of lysotracker green in MCF7/FP cells was due to elevated levels of an ATP-dependent transporter activity, and not a consequence of reduced dye uptake, MCF7 and MCF7/FP cells were pretreated with sodium azide and deoxyglucose to deplete cells of ATP. The cells were then exposed to dye for 30 minutes, and confocal images collected. Figure 8 shows fluorescence micrographs of the two cell lines following exposure to lysotracker green under control and ATP depletion regimes. The intensity of fluorescence in MCF7 cells was not affected by the ATP depletion protocol. In contrast, MCF7/FP treated with the ATP-depleting agents had greatly reduced intracellular concentrations of lysotracker green. This finding indicates that MCF7/FP cells rely upon an energy dependent process to reduce intracellular lysotracker green concentrations.

DISCUSSION

In this report we describe the isolation and characterization of the cell line MCF7/FP which is 60-fold less sensitive to the cytotoxic effect of FP than the parental MCF7 cell line. These cells, which emerged from a protocol in which cells were serially passaged in gradually increasing concentrations of FP, are 2.5-fold less sensitive than their parental cell line to killing induced by mitoxantrone. In contrast, the MCF7/FP cells are no more resistant to cisplatin, paclitaxel, vinblastine, or topotecan than is the parental line. Western blot analysis revealed wild-type expression levels of cdks 1,2,4,7,8 and 9, and of cyclins A, B, D3 and E. The MCF7/FP cells had slightly decreased levels of cyclin D1 expression, compared to the parental MCF7 cells. ABCG2 protein levels are elevated three-fold above control levels in MCF7/FP cells, while levels of p-glycoprotein are unaltered.

The finding that cyclin D1 expression is reduced in MCF7/FP cells was unanticipated. Furthermore, it is difficult to imagine how diminished cyclin D1 expression could contribute to the FP resistance of MCF7/FP cells. It is known, however, that FP interferes with transcriptional activation of the cyclin D1 promoter (30). This agent was not present when the MCF7/FP cells were expanded, nor at the time they were harvested to make the protein extracts used for the western blot analysis However, it is conceivable that the consequence of long term exposure to FP (the process of generating the MCF7/FP cell line consumed nearly a year) may have resulted in a constitutive partial down regulation of the transcriptional activity of the cyclin D1 promoter. In any event, we do not believe that the diminished levels of cyclin D1 play a role in the FP resistance observed in the MCF7/FP cell line.

Of potentially greater interest is the finding of elevated expression of the drug efflux pump ABCG2 in these cells. Robey *et al.* (20) described a FP-resistant cell line, MCF7 FLV1000, that was derived from the human breast cancer line MCF7 using a selection protocol nearly identical to that utilized to isolate MCF7/FP. Interestingly, both MCF7 FLV1000 and MCF7/FP cell lines display elevated levels of the ABCG2 transporter. Furthermore, Robey *et al.* (20) showed that the FP resistance observed in MCF7 FLV1000 cells was entirely reversed by 5μM fumitremorgin C, a specific inhibitor of the ABCG2 transporter, and that transfectant cells that overexpress the ABCG2 gene are also resistant to FP. Taken together, these findings suggest that a common mechanism, i.e. enhanced efflux of FP via the ABCG2 protein, may underlie the FP resistance of the MCF7 FLV1000 and MCF7/FP cell lines. However, MCF7 FLV1000 cells display tremendous cross-resistance to mitoxantrone (675-fold more resistant than MCF7) and topotecan (423-fold more resistant than MCF7), and are 3.6-fold more resistant to paclitaxol than are MCF7 cells. In contrast, MCF7/FP cells do not display increased resistance to either topotecan or paclitaxol, and are only about 2.5-fold more resistant to mitoxantrone than are MCF7 cells. Furthermore, while ABCG2 protein expression levels in MCF7 FLV1000 cells are nearly 50-fold greater than those present in MCF7 cells, MCF7/FP cells express only about three-fold more ABCG2 protein than do MCF7 cells. Robey *et al.* (20) showed that MCF7 cells displaying modest overexpression of ABCG2, such as is present in the MCF7/FP cell line, are not resistant to high concentrations of FP. Finally, while the FP resistance observed in MCF7 FLV1000 cells was entirely reversed by 5μM fumitremorgin C, this agent had no effect of the FP resistance observed in the MCF7/FP cells. It therefore appears unlikely that

the FP resistance in MCF7/FP cells can be satisfactorily explained by the modestly increased expression level of the ABCG2 transporter observed in these cells, although this could play a role in the resistance of these cells to mitoxantrone, since exposure to fumitremorgin C partially sensitized these cells to this agent (data not shown).

Recent evidence has shown that mutant variants of the ABCG2 gene carrying certain mutations encode transport proteins that possess an altered substrate specificity with respect to cross resistance and efflux of certain drugs (31). It is therefore formally possible that the drug resistance pattern in MCF7/FP cells could result from low level over expression of such an allele of the ABCG2 protein. We think this conclusion, while plausible, is unlikely for the following reasons. First, in addition to possessing an altered specificity, the mutation or mutations within the ABCG2 protein would also have to significantly enhance the affinity of the transporter for FP. As has been mentioned above, high levels of drug resistance have not been previously associated with low level overexpression of the ABCG2 transporter. Second, and more significantly, the available evidence indicates that all of the previously characterized variants of ABCG2 are all inhibited by fumitremorgin C (32). Since the FP resistance phenotype of MCF7/FP cells is unaffected by the presence of fumitremorgin C, it would be necessary to propose that the mutant allele of ABCG2 that confers resistance to MCF7/FP cells also encodes a fumitremorgin C-resistance. Given that the cells were never exposed to fumitremorgin C during the generation of their drug-resistance phenotype, this would seem unlikely. We therefore propose that a novel ATP-dependent drug efflux pump is responsible for the FP resistance in these cells.

It is conceivable that the FP resistance of MCF7/FP cells is associated with whatever mechanism is responsible for the high efflux of lysotracker green from these cells. In other words, it is possible that both FP and lysotracker green are rapidly effluxed from MCF7/FP cells via a common mechanism. If this is true, however, this transporter does not apparently efflux any of a variety of other compounds such as cisplatin, paclitaxel, vinblastine or topotecan, based on cross resistance patterns observed in MCF7/FP cells. It is unclear whether this transporter contributes to marginal cross resistance to mitoxantrone in MCF7/FP cell line, since these cells retain a slightly elevated resistance to this compound, even in the presence of fumitremorgin C (data not shown). Alternately, other mechanisms of drug-resistance such as involvement of more than one transporter or gross mutations in an existing transporter cannot be completely ruled out. We are currently using gene transfer techniques in an effort to clone the gene or genes responsible for conferring FP resistance on MCF7/FP cells.

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FIGURE LEGENDS

Figure 1: Sensitivity of MCF7 and MCF7/FP cells to flavopiridol. Clonogenic assays were carried out as described in Materials and Methods. Open circles represent MCF7 cells and closed circles represent MCF7/FP cells. The values represent a mean of five independent experiments. Error bars depict the standard error of the mean.

Figure 2: CDK levels in MCF7 and MCF7/FP cells

(A) Western blots were performed on 30 µg of whole cell protein extract prepared from MCF7 and MCF7/FP cells using specific antisera as indicated. The mobility of the respective cdks, and their respective molecular masses are indicated.

(B) Western blot analysis was performed on three independently prepared sets of whole cell extracts from MCF7 and MCF7/FP cells. Scanning densitometry was used to quantify the intensity of bands corresponding to the respective cdk proteins. From each set of extracts, the relative cdk protein expression level in MCF7/FP cells was divided by that present in MCF7 cells. The graphs depict the average expression level of the various cdk proteins in MCF7/FP cells relative to that detected in MCF7 cells. Error bars depict the standard error of the mean.

Figure 3: Analysis of cyclin levels in MCF7 and MCF7/FP cells

(A) Western blots were performed on 30 µg of whole cell protein extract prepared from MCF7 and MCF7/FP cells using specific antisera as indicated. The mobility of the respective cyclin proteins, and their respective molecular masses are indicated.

(B) Relative levels of cyclin protein expression in MCF7/FP and MCF7 cells were determined as described in the legend to Figure 2B.. Error bars depict the standard error of the mean.

Figure 4: ABCG2 expression in MCF7 and MCF7/FP cells

(A) Northern blot analysis was performed on 10 mg of the total RNA isolated from MCF7 and MCF7/FP cells using probes specific for GAPDH or ABCG2 (Left Panel). Scanning densitometry was used to determine the ratio of mRNA levels in the two cell lines as described in the legend to Figure 2B, and the graph depicts the mean ABCG2 mRNA expression level MCF7/FP relative to that in present in MCF7 cells. Error bars depict the standard error of the mean.

(B) Western blot analysis was performed on 30 µg of whole cell extracts using antisera specific for ABCG2 and P-glycoprotein. The mobilities of the P-glycoprotein (pGP) and ABCG2 proteins are indicated by arrows, and their molecular weights provided (Left Panel). Western blot analysis was conducted on three independent extracts and the relative intensities of the respective proteins determined by scanning densitometry as described above. Error bars depict the standard error of the mean.

(C) Immunohistochemical analysis: Cellular localization of ABCG2 protein in MCF7 and MCF7/FP cells was determined using an antibody specific for the ABCG2 protein as described in the Materials and Methods section.

Figure 5: Determination of cross-resistance patterns of MCF7 and MCF7/FP cells

Clonogenic assays were performed as described in the Materials and Methods section using the indicated drugs for MCF7 cells (open circles) and MCF7/FP cells (Closed

circles). Data represent the mean of three independent experiments, and the error bars depict the standard error of the mean.

Figure 6: Fumitremorgin C does not sensitize MCF7/FP cells to FP.

Cytotoxicity assays were performed on MCF7 cells (open circles), and on MCF7/FP cells in the absence (filled circles) or presence (filled triangles) of 5 mM fumitremorgin C. Data represent the average of two or more experiments. Error bars depict the standard error of the mean.

Figure 7: Intracellular accumulation of fluorescent dyes in MCF7 and MCF7/FP cells. MCF7 and MCF7/FP cells were incubated with calcein AM, lysotracker green or rhodamine 123 for 30 min at 37 °C. Cells were washed with serum free media, suspended in serum-containing media, and images immediately obtained using a confocal fluorescence microscope.

Figure 8: Efflux of lysotracker green from MCF7/FP cells is ATP-dependent

MCF7 and MCF7/FP cells were pretreated with sodium azide and deoxyglucose for 1 h at 37 °C to deplete the cells of ATP. Untreated cells were used as control. Cells were subsequently incubated with lysotracker green for 30 min at 37 C, and images immediately obtained using a confocal fluorescence microscope.

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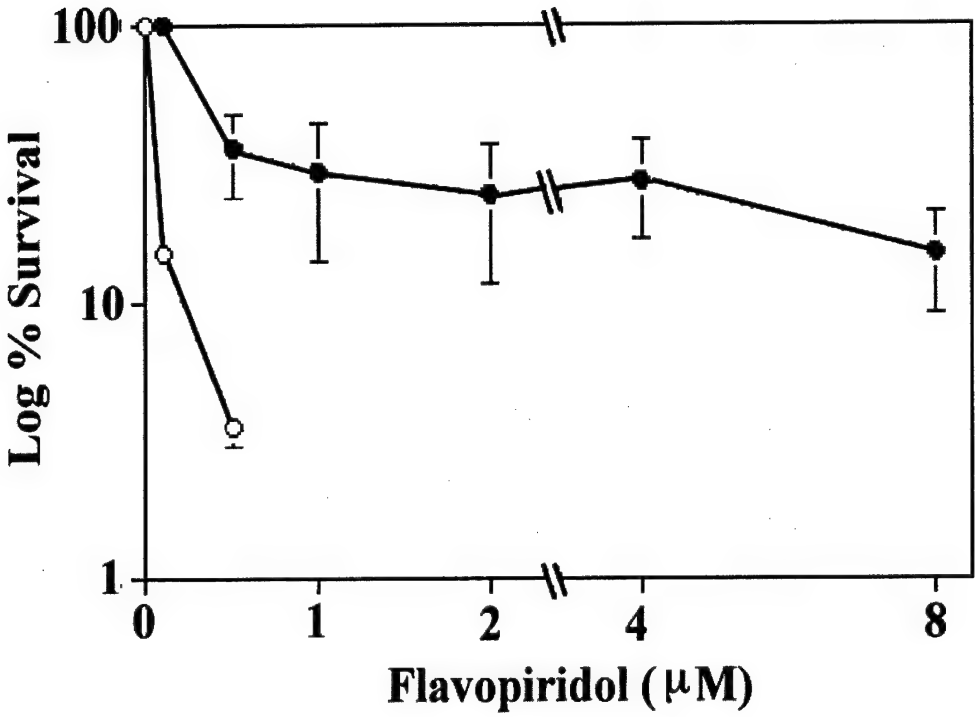
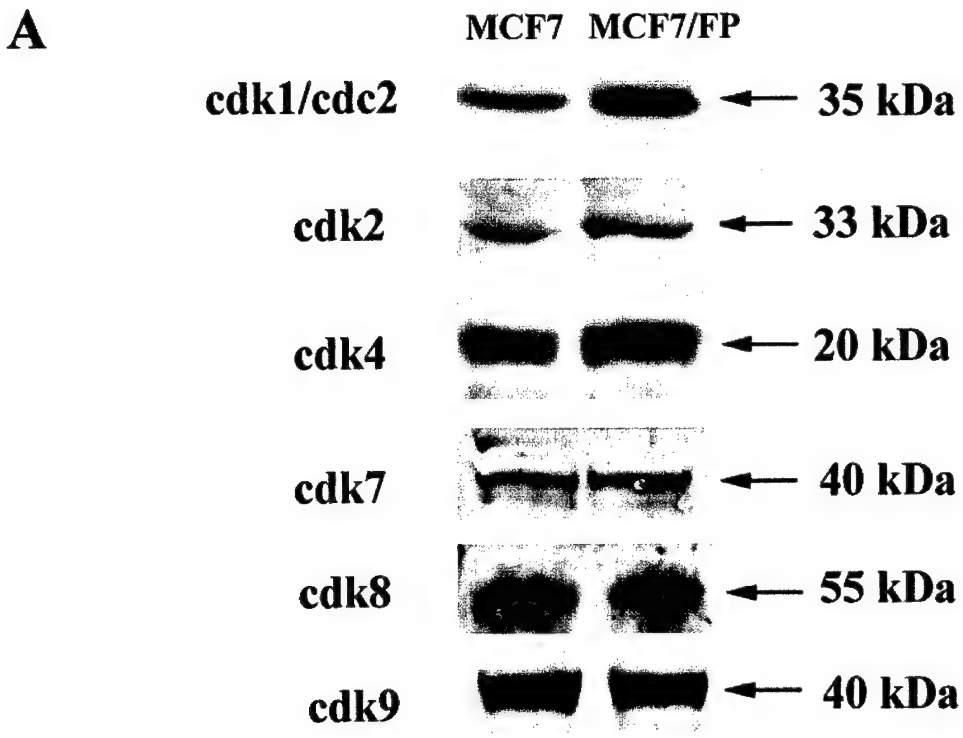


Figure 1


A
MCF7
MCF7/FP
cdk1/cdc2
35 kDa
cdk2
33 kDa
cdk4
20 kDa
cdk7
40 kDa
cdk8
55 kDa
cdk9
40 kDa

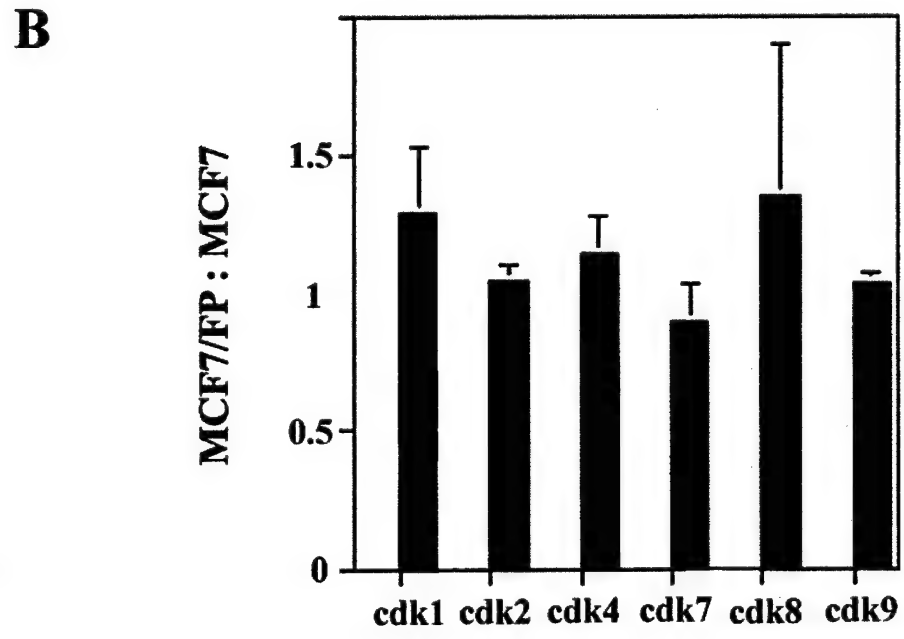
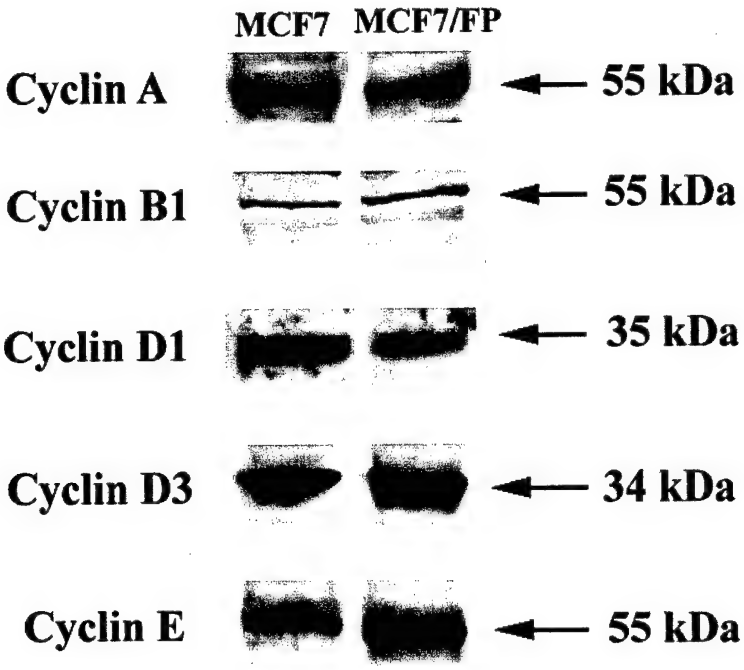

B
MCF7/FP : MCF7
1.5
1
0.5
0
cdk1
cdk2
cdk4
cdk7
cdk8
cdk9

Figure 2

A


MCF7
MCF7/FP
Cyclin A
55 kDa
Cyclin B1
55 kDa
Cyclin D1
35 kDa
Cyclin D3
34 kDa
Cyclin E
55 kDa

B

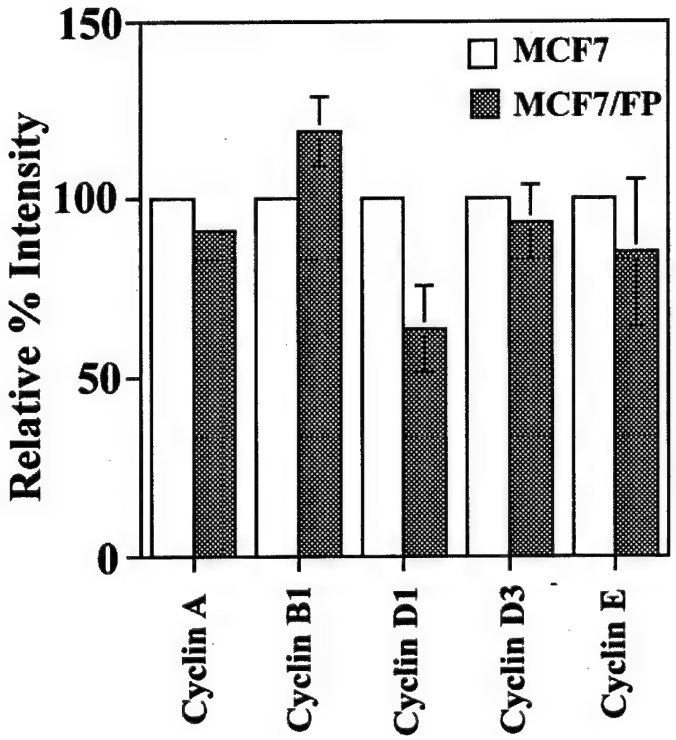

150
100
50
0
Relative % Intensity
MCF7
MCF7/FP
Cyclin A
Cyclin B1
Cyclin D1
Cyclin D3
Cyclin E

Figure 3

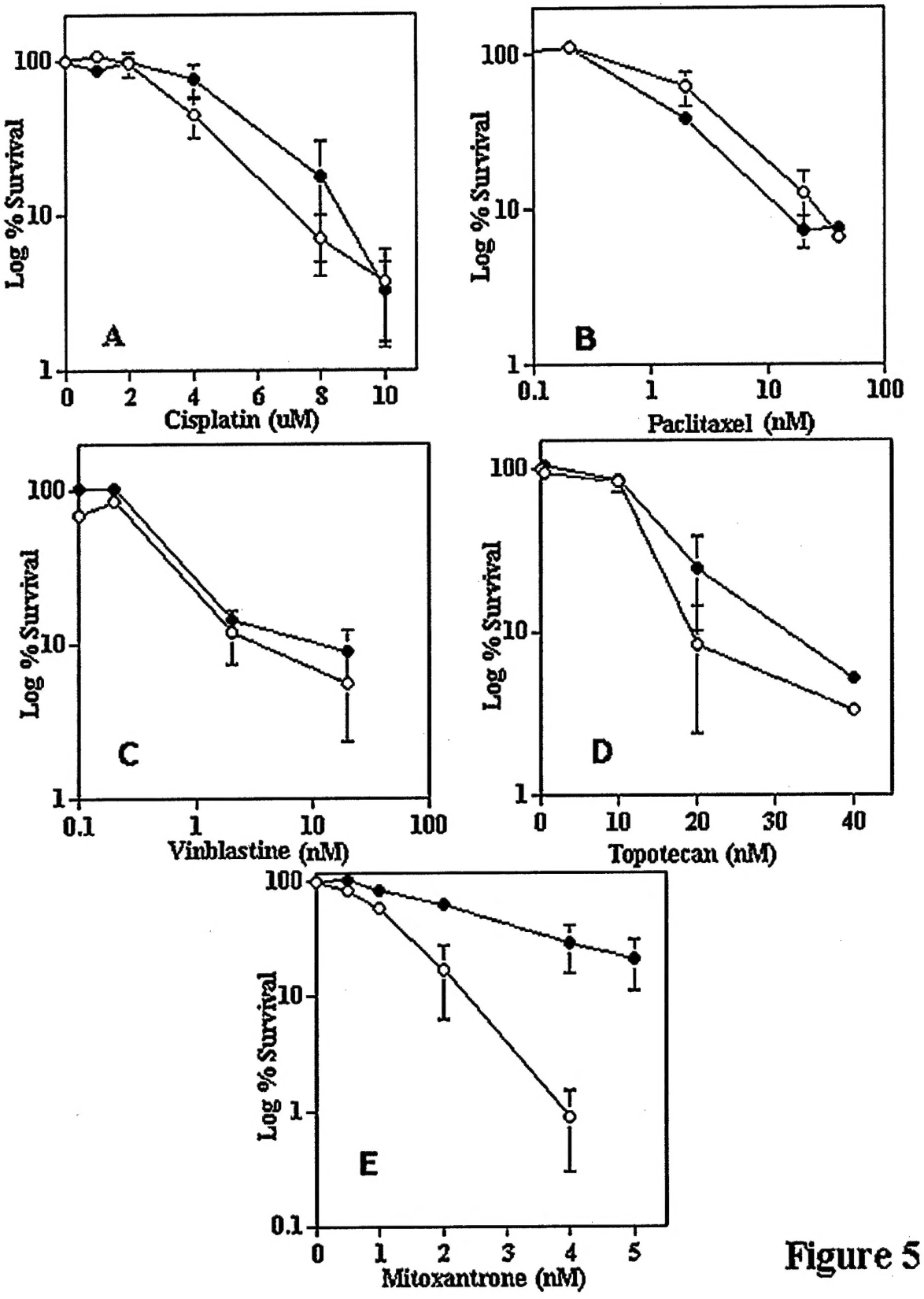

Log % Survival
100
10
1
A
0
2
4
6
8
10
Cisplatin (uM)
B
0.1
1
10
100
Paclitaxel (nM)
C
Vinblastine (nM)
D
20
30
40
Topotecan (nM)
E
0.1
3
5
Mitoxantrone (nM)

Figure 5

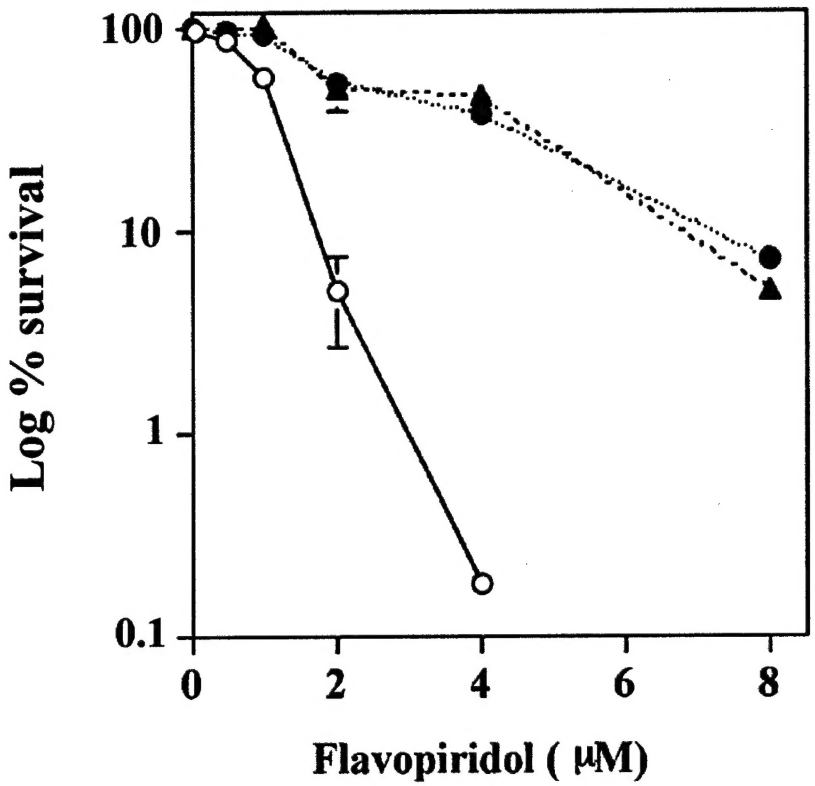


Figure 6

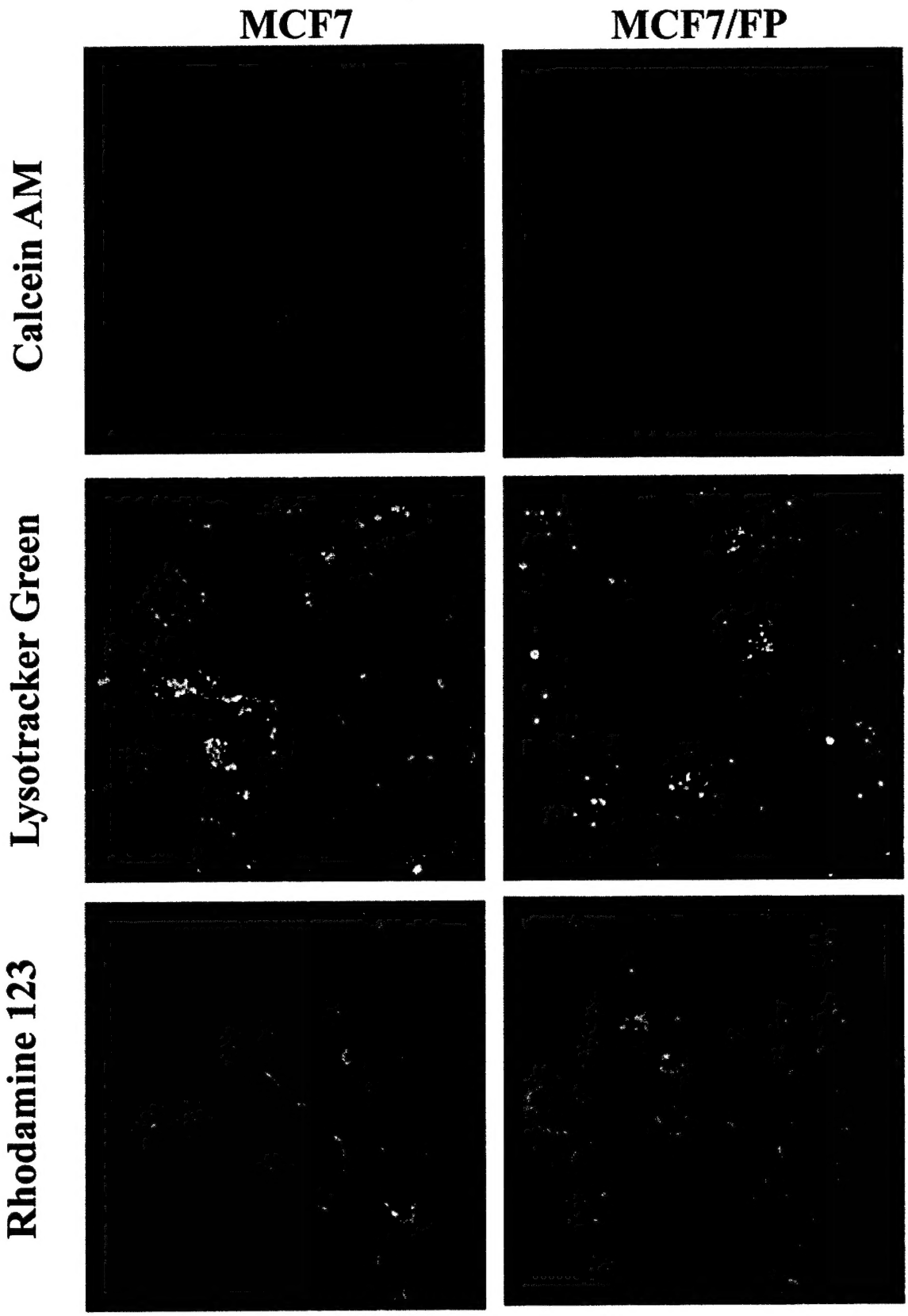


Figure 8

GENERATION AND CHARACTERIZATION OF AN MCF7 CELL LINE RESISTANT TO FLAVOPIRIDOL WITH A UNIQUE PATTERN OF CROSS-RESISTANCE

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Flavopiridol is a potential anti cancer drug with a novel mechanism of action via inhibition of cyclin dependant kinases (CDKs). Several studies report the emergence of flavopiridol-resistance in cells, thereby necessitating a thorough understanding of the various mechanisms involved in flavopiridol-resistance. To achieve this objective, we developed a flavopiridol-resistant cell culture model by serially growing breast cancer MCF7 cells in increasing concentrations of drug. A single clone, MCF7/FP, resistant to high concentrations of flavopiridol (IC90 > 8 μM) relative to the parental MCF7 cells (IC90=0.3 μM), was isolated.

Western blot analysis of protein extracts prepared from MCF7/FP and parental MCF7 cells was carried out to determine changes in expression levels of CDKs. The expression level of six different cdks and 5 cyclins, proteins known to be involved in cell cycle regulation, did not differ between the two cell lines. In addition, the cell cycle kinetics of the two cell lines were indistinguishable.

Studies from other labs have reported that cells with acquired resistance to flavopiridol have elevated levels of ABCG2, the principal drug transporter responsible for efflux of flavopiridol and other drugs such as topotecan and mitoxantrone. To examine if MCF7/FP cells had a similar mechanism of flavopiridol-resistance, the expression level of ABCG2 in MCF7/FP and MCF7 cells was measured. Intriguingly, a mere three-fold increase in the mRNA and protein levels of ABCG2 was observed in MCF7/FP cells relative to the parental MCF7 cells. Since the extent of ABCG2 overexpression has been shown to correspond to the level of resistance to flavopiridol, the modest increase in ABCG2 expression cannot readily explain the high levels of flavopiridol-resistance seen in MCF7/FP cells. Furthermore, these cells showed only marginally elevated levels of resistance to mitoxantrone with no cross reactivity to topothecan.

These results support the conclusion that cellular resistance to flavopiridol can arise through a uncharacterized novel mechanism. This finding suggest that issues of flavopiridol-resistance in the clinic may represent a greater challenge than was originally hoped, highlighting the need to understand these processes in greater detail.

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